EXPERIMENTAL

Isolation of veratric acid. Some 800 oothecae of *Blatta orientalis* were crushed in dilute aqueous potassium cyanide (*c.* 250 ml.) containing some sulphur dioxide. After 3 hr. the mixture was filtered with the aid of 'Hyflo supercel'. The filtrate was weakly acid to litmus. The residue was extracted further by allowing to stand for 2 hr. with water (300 ml.) which had been made just acid to Congo red with sulphuric acid, and the procedure repeated until the extracts no longer gave a green colour with ferric chloride. The combined filtrate and extracts were concentrated *in vacuo* under nitrogen to small bulk (*c.* 300 ml.), the concentrate filtered from a trace of solid material and extracted continuously with peroxide-free ether (700 ml.). The ethereal extract which contained all the material giving a coloration with ferric chloride was concentrated to one-third bulk and treated with ethereal diazomethane (from 10 g. nitroso-*N*-methylurea). After 2 days the ether was evaporated and the residue refluxed for 2 hr. with methanolic potassium hydroxide (2 %). Removal of methanol, dissolution in water and acidification of the aqueous solution gave brownish needles. Recrystallized from water this material formed colourless needles, m.p. 179–180·5°. (Found: C, 59·2; H, 5·6. Calc. for $C_9H_{10}O_4$: C, 59·3; H,

5·5%.) A mixed m.p. with authentic veratric acid (m.p. 180–181°) showed no depression. For further identification the isolated acid was converted via its acid chloride to the anilide; flat needles, m.p. 168·5°, undepressed in admixture with a specimen, m.p. 169°, prepared in similar fashion from authentic veratric acid. (Found: C, 70·2; H, 5·9; N, 5·5. Calc. for $C_{15}H_{15}O_3N$: C, 70·0; H, 5·7; N, 5·5%.) Veratric anilide is described by Bruggemann (1896) as having m.p. 154°, but this appears to be an error.

Further small amounts of product were obtained from the aqueous mother liquors after removal of the precipitated acid from the hydrolysis solution. The total yield of isolated veratric acid was 426 mg.

Isolation of protocatechuic acid. Approximately 1100 oothecae were extracted as above described, but instead of treating the ethereal solution of the phenolic material with diazomethane it was evaporated to dryness. The brownish residue recrystallized from water (charcoal) gave needles of protocatechuic acid (309 mg.), m.p. 198°, undepressed in admixture with an authentic specimen (m.p. 197·5°).

SUMMARY

The phenolic substance present in cockroach oothecae has been isolated and identified as protocatechuic acid (3:4-dihydroxybenzoic acid).

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The Spectrophotometric Determination of Tyrosine and Tryptophan in Proteins

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Solutions of most proteins show selective absorption in the ultra-violet. In each case the exact position of the maximum (near 280 mμ) varies with pH. The majority of the constituent amino-acids are practically transparent in the region 250–320 mμ and it is known that phenylalanine, tyrosine and tryptophan are together responsible for the observed absorption of protein solutions. Phenylalanine, as would be expected from its constitution, shows weak absorption, which under favourable conditions

can be resolved into several narrow bands, but in 0·1 N-NaOH the maximum occurs near 258·5 mμ with a low molecular extinction coefficient (*c.* 200). The absorption exerted by tyrosine and by tryptophan is much stronger, and as a first approximation the phenylalanine contribution can be neglected. Inasmuch as wave-lengths can be selected at which phenylalanine makes no contribution, it should be possible to treat protein solutions as two-component systems for spectrophotometric analysis.

Vierordt (quoted by Twyman & Allsopp, 1934) has described a procedure for analyzing binary systems by using intensities of absorption at selected wave-lengths. The method can be extended to protein solutions and hydrolysates if it is assumed that neither the mode of linkage nor the presence of other amino-acids significantly distorts or displaces the tyrosine-tryptophan absorption. The same assumption is necessary for any alternative to Vierordt's procedure, as otherwise a whole series of corrections, depending on the order in which amino-acids are joined together, would be inevitable. Fortunately, the assumption seems to be justified. Thus the dipeptide glycyltyrosine shows the absorption expected of its tyrosine moiety (Abderhalden & Haas, 1927); but in fact the true grounds for accepting the assumption are:

(a) it can be foreseen on the basis of much experience on organic chromophores in general;

(b) it leads to estimates of tyrosine and tryptophan agreeing closely with those obtained by other methods.

There have been very few investigations in which a sufficiently refined technique has been available for the detection of very small displacements. Summarizing one such study Coulter, Stone & Kabat (1936) state: 'The bands in the proteins are accordingly attributed to these amino-acids (phenylalanine, tyrosine, tryptophan). In the proteins the bands are displaced from the positions which they occupy in the uncombined amino-acids, in most instances, by 10–35 A. towards longer wave-lengths.' These authors used neutral aqueous solutions or slightly acid solutions, and as they did not study the distribution of the absorption between tyrosine and tryptophan and made no corrections for irrelevant absorption, the appropriate conclusion from their work is that no displacement due to peptide linkage is demonstrable.

Holiday (1936) used the spectrophotometric method with considerable success. By determining intensities of absorption at 280 and 305 mμ, he was able to analyze mixtures of tyrosine and tryptophan

tions. Another, if less marked, source of error arose from the occurrence of variable irrelevant general absorption.

Our own work in this field was begun in 1938, when we were aiming at the elimination of errors due to irrelevant absorption. Partial success was achieved by working with protein hydrolysates (Morton, 1942), and the best results were obtained by alkaline hydrolysis in presence of stannite (Lugg, 1938), but the lengthy preliminaries robbed the spectroscopic method of simplicity and rapidity compared with chemical methods. The incentive to persist, however, remained, inasmuch as very small samples of protein (c. 25 mg.) could be analyzed provided that irrelevant absorption could be correctly assessed. To this end a substantial increase in accuracy in the determination of intensities of absorption at sharply defined wave-lengths was imperative. The Beckman photoelectric spectrophotometer (Cary & Beckman, 1941) represents a considerable advance in this direction, and we have used the instrument to make a fresh approach to the task.

METHODS AND RESULTS

The protein solvent has throughout been 0·1 N-NaOH. It is necessary to work at a fixed pH and the considerations which led Holiday (1936) to choose decinormal alkali are still valid. The absorption cells were 1 cm. wide and the solvent was always used for compensation. Concentrations were adjusted so as to maintain E values between 0·3 and 0·8, over which range the Beckman instrument gives accurate and highly reproducible results. When E values were required for wave-lengths between dial calibrations (e.g. 257 and 258 mμ) they were obtained by interpolation.

Absorption spectra of tyrosine and tryptophan. The values given in Table 1 were obtained on 3 samples of tryptophan and 4 of tyrosine. At no point were differences recorded greater than ± 1 % from the means.

Table 1. *Molecular extinction coefficients in* 0·1 N-NaOH

λ mμ	250	255	257	258	260	270	280	290	294	295	305
Tyrosine	6640	3726	2811	2448	1882	1054	1576	2377	2389	2360	1246
Tryptophan	1946	2580	2730	2910	3188	4644	5225	4130	2531	2176	420

	λ max. mμ	ϵ max.
Tyrosine	293	2390
Tryptophan	280·5	5250

dissolved in 0·1 N-NaOH within ± 4 and ± 8 % respectively. The limiting factor was the accuracy with which the blackening of a photographic plate could be estimated, and later (Holiday & Ogston, 1938) he effected improvement by substituting photoelectric microphotometry for visual determina-

Determination of tyrosine and tryptophan. The characteristics of the absorption curves for tyrosine and tryptophan suggest a modification of Vierordt's procedure for calculating the composition of binary mixtures of solutes. If the two curves intersect at one or more points, the intensity of absorption is a

direct measure of the total molar solute concentration, and will be the same however the proportions are varied. At other wave-lengths the intensity of absorption will vary with the relative proportions of the components. Using 0·1 N-NaOH as solvent the two absorption curves intersect at

294·4 mμ (ε, 2375)   and   257·15 mμ (ε, 2748).

In a simple mixture the sum of the molar concentrations of tyrosine and tryptophan may be obtained from measurements of intensity of absorption at either wave-length. The 294·4 mμ point is the more suitable because it lies near the maximum in the tyrosine curve (Δε/Δλ minimal). In using points of intersection rather

ditioned by the use of a single rather than a double monochromator are thus minimized.

Calibration. Mixtures of tyrosine and tryptophan were prepared in which molar ratio of the two solutes varied widely. The analyses were completed before the proportions were disclosed.

Of the two intersections, that at 294·4 mμ gave better results than that at 257·15 mμ. This is not surprising, because at the latter point both absorption curves slope steeply, whereas the former, as stated above, is near the peak of the tyrosine absorption and Δε/Δλ is minimal.

The results in Table 2 were obtained by combining the results at 294·4 mμ with those at 280 mμ (but 270 and 290 mμ gave equally good results).

Table 2. *Tyrosine-tryptophan mixtures*

	Tyrosine			Tryptophan			Molar ratio tyrosine/ tryptophan
Solution	Taken (M)	Found (M)	'Recovery' (%)	Taken (M)	Found (M)	'Recovery' (%)	
1	0·0833	0·0864	103·7	0·0784	0·0798	101·9	1·06
2	0·1250	0·1244	99·9	0·0588	0·0586	99·7	2·11
3	0·0500	0·0525	105·0	0·0941	0·0952	101·2	0·53
4	0·2083	0·2079	99·8	0·0196	0·0178	91·0	10·62
5	0·1667	0·1654	99·3	0·0167	0·0162	96·9	10·00
6	0·0095	0·010	105·2	0·0905	0·0906	100·1	0·11
7	0·0400	0·0379	95·0	0·0800	0·0779	97·4	0·50
8	0·180	0·1925	106·2	0·0047	0·0017	36·1	38·5
9	—	0·0001	—	0·100	0·0960	96·0	—
10	0·006	0·0167	278·3	0·104	0·100	96·1	0·0576
11	0·2268	0·2258	99·6	0·0097	0·0112	115·4	23·4
12	0·2500	0·2480	99·2	—	—	—	—
13	0·2368	0·2336	98·6	0·0056	0·006	106·2	42·1
14	—	0·0007	—	0·1072	0·1049	97·7	—

than following Vierordt's method, the monochromator must be efficient and the intensities of absorption must be accurately measurable. Granted adequate technique it is an undoubted advantage first to determine total molar concentration. An accurate reading at one other wave-length is then sufficient to determine the relative proportions of the two solutes.

Thus, if x = total mol./l. in solution, y = g.mol. of tyrosine, $x - y$ = g.mol. of tryptophan. At any wave-length other than a point of intersection let

ε tyrosine be A,   ε tryptophan be B,

and the observed intensity of absorption for a 1 cm. cell E

$$E = yA + (x - y) B, \quad y = \frac{E - xB}{A - B}$$

(x determined from the E value at an intersection).

In selecting 280 mμ as the wave-length to be used in conjunction with 294·4 mμ a departure has been made from the choice arrived at by Holiday (1936) for the reason that Δε/Δλ is minimal at that point for tryptophan. Instrumental limitations con-

The range of concentrations covered by Table 2 is a very wide one, and constitutes a very severe test of the technique. The intersection at 294·4 mμ would give substantially the same E value for a solution in which tyrosine predominated, whether the slit were very narrow or fairly wide because $\Delta E/\Delta\lambda$ is small, whereas on the steeply rising curve for solutions in which tryptophan predominates the slit width and resolving power are more critical. The Beckman instrument stands up well to this test, but an instrument embodying a double monochromator could doubtless have given even better results. Thus solutions 9 and 14, which actually contained no tyrosine, were both reported as practically free from that substance, but the absolute concentration of tryptophan was slightly under-estimated.* Solutions 8, 10 and 11 with molar ratios > 20 : 1 gave inaccurate results for the minor component. It would seem that the limitations of the method are only significant at such extreme ratios.

* If the analysis indicates absence of tyrosine, tryptophan is more accurately determined at its maximum (280·5 mμ).

Estimation of tyrosine and tryptophan in protein solutions

Allowance for irrelevant absorption. Examination of the spectra of protein solutions (in 0.1N-NaOH) reveals general absorption falling off with increasing wave-length, but persisting into the region 330–400 mμ where tyrosine and tryptophan are transparent. In order to allow for irrelevant absorption at 294·4 and 280 mμ, the simplest procedure is to extrapolate linearly from 370 and 340 mμ to those wave-lengths, e.g.

$$\left.\begin{array}{l} E\ 370\ \text{m}\mu = 0.020 \\ E\ 340\ \text{m}\mu = 0.028 \end{array}\right\} \quad \begin{array}{l} \Delta\ \text{m}\mu = 30, \quad \Delta E = 0.008 \\ \\ \Delta\ \text{m}\mu / \Delta E = \dfrac{30}{0.008} \end{array}$$

expected

$$\left.\begin{array}{l} E\ 294\ \text{m}\mu = 0.040 \\ E\ 280\ \text{m}\mu = 0.044 \end{array}\right\} \quad \text{for the irrelevant absorption.}$$

The validity of the extrapolation may best be demonstrated by comparing tyrosine and tryptophan values obtained by using the extinction at the crossing point (294·4 mμ) in conjunction with extinctions at different wave-lengths. The uncorrected extinctions will not give consistent results because of the varying amounts of irrelevant absorption contributing to the gross E values at the different wave-lengths. If, after correcting for irrelevant absorption, concordant results are obtained, it must be assumed that this absorption has been eliminated. The results obtained from an experiment carried out on a solution of casein (Table 3) show that the corrections applied must be near the truth and justify the extrapolation over the wave-length range normally employed.

Table 3. *Tyrosine and tryptophan values of a casein solution obtained using E values at a crossing point (294·4 mμ) and at one other wave-length*

Other wave-length used (mμ)	Tyrosine content of solution (mM)	
	'Uncorrected'	Corrected
275	143	130
280	136	137
285	160	138
290	164	139

The results quoted in Table 4 will illustrate that accuracy of measurement in the spectrophotometer is adequate to deal with the differences in E values which this correction procedure requires. To solutions of zein and gelatin, both of which proteins show considerable irrelevant absorption, were added small amounts of tyrosine and tryptophan. The recovery of these added acids is satisfactory even under the severe conditions chosen for this experiment.

Table 4. *Analyses of zein and gelatin with and without added amino-acids*

	Amino-acids of protein (M)	Recovery in amino-acid-protein mixture*	
		Expected (M)	Found (M)
(i) Zein			
Tyrosine	0·1314	0·1132	0·1172
Tryptophan	Nil	0·0242	0·0263
(ii) Gelatin			
Tyrosine	0·0397	0·0674	0·0700
Tryptophan	0·0066	0·0275	0·0292

* 10 ml. of protein solution; 5 ml. of 0·19 M-tyrosine; 5 ml. of 0·0964 M-tryptophan.

All the foregoing experience leads to the conclusion that there is little to be gained by examining hydrolysates rather than simple solutions of proteins. Indeed, in many instances, irrelevant absorption rises on hydrolysis and there is always a risk of destroying some, at least, of the tryptophan.

Up to this point attention has been focused upon the limitations of the analytical procedure. To illustrate reproducibility, the results on casein may be quoted (Table 5). In this protein the relative proportions of tyrosine and tryptophan favour good results and irrelevant absorption is small.

Table 5. *Analysis of casein**

Experiment ...	A	B	C
Tyrosine (%)	5·47$_4$	5·48$_2$	5·48$_5$
Tryptophan (%)	1·60$_7$	1·62$_6$	1·62$_6$

* Samples of commercial preparations were taken from airtight containers. Figures uncorrected for moisture content.

Table 6. *Estimated tyrosine and tryptophan in various proteins*

	Tyrosine (%)	Tryptophan (%)
Casein	5·48	1·62
Zein	5·15	None found
Gelatin	0·42	0·06
Ovalbumin	3·50	1·33
Edestin	5·18	1·19
Bence Jones protein	6·57	2·33
Insulin (crystalline)	11·55	0·18*

* The tyrosine predominates over the tryptophan to an extent which reduced the significance of this result.

Various proteins have finally been analyzed by the method suggested and the results are collected in Table 6. They fall well within the rather wide range of values reported in chemical and micro-biological assays. Our main object throughout this investigation was to prove the speed and accuracy of the method for analyzing small amounts of proteins, and was not primarily to determine the

absolute values of tyrosine and tryptophan in 'pure' proteins. The proteins quoted in Table 6 are normal commercial products and no attempt has been made to assess their purity.

SUMMARY

1. The ultra-violet absorption spectra of tyrosine and tryptophan have been determined in 0·1 N-NaOH and the curves found to intersect at 257·15 and 294·4 mμ (ϵ, 2748 and 2375, respectively).
2. By using intensities of absorption at 294·4 and 280 mμ, determined by means of a photoelectric spectrophotometer, mixtures of tyrosine and tryptophan may be analyzed with considerable accuracy provided the molar ratios are not greater than 20 : 1 either way.
3. Using about 25 mg. of material, proteins may be analyzed with comparable accuracy if due correction is made for irrelevant continuous absorption.

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The Estimation of Threonine and Serine in Proteins

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Although serine was discovered by Cramer as long ago as 1865, its isolation by the older gravimetric methods was so laborious and intractable, except perhaps from the proteins of silk, that the values recorded in the literature for the amounts present in other proteins were no more than minimal estimates. Threonine, discovered as late as 1935 by McCoy, Meyer & Rose, was likewise in the same category; feeding experiments showed that its presence in proteins must be widespread, but quantitative data were entirely lacking. As a consequence, the new and indirect method introduced by Nicolet & Shinn (1939) for the estimation of these two hydroxy-amino-acids has attracted attention.

Nicolet & Shinn showed that β-hydroxy-α-amino-acids react with periodates. Serine and threonine give ammonia, glyoxylic acid and a free aldehyde, and under suitably chosen conditions the reaction is quantitative and practically instantaneous. The formaldehyde and acetaldehyde thus formed were made the basis for the determination of serine and threonine in protein hydrolysates.

Shinn & Nicolet (1941) found that the acetaldehyde produced from threonine is carried over by aeration at a pH close to 7, and preferably pH 7·0–7·2. In the case of protein hydrolysates, the formaldehyde produced from the serine does not cause contamination as it is held back by the large excess of primary amino groups present; with relatively pure solutions of serine and threonine the tendency for inconvenient amounts of formaldehyde to volatilize may be sufficiently restrained by the addition of an adequate excess (10 mol. or more) of alanine. In their procedure for the estimation of threonine, the periodate reaction is carried out in the presence of a bicarbonate buffer and the pH maintained within the suggested range by aeration with CO_2. The acetaldehyde is absorbed in sodium bisulphite solution and a subsequent titration gives a measure of threonine. As a routine procedure in protein analysis they admit that such evidence is not specific for acetaldehyde but, according to their experience, treatment of the bisulphite solution to yield again the aldehyde for precipitation with dimedone (Vörlander, 1929) always gave an approximately pure dimedone derivative. Martin & Synge (1941), exploring the possible use of the periodate reaction for the same purpose, could find no evidence for the presence of higher homologues of acetaldehyde, and hence of higher homologues of threonine, on treatment of a number of protein hydrolysates.

Continuing their studies, Nicolet & Shinn (1941a) found that the formaldehyde produced from serine could be determined as the dimedone derivative by adding the reagent to the residual acetaldehyde-free solution. From known mixtures, the recovery of serine ranged from 95 to 98%. Meanwhile Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) had made use of the ammonia produced in the periodate reaction in their investigations on the structure of hydroxylysine, and had incidentally reported